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RESEARCH ARTICLE

Effect of nontoxigenic *Aspergillus flavus* and *A. parasiticus* on aflatoxin contamination of wounded peanut seeds inoculated with agricultural soil containing natural fungal populations

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Peanuts and other seed and grain crops are commonly contaminated with carcinogenic aflatoxins, secondary metabolites produced by *Aspergillus flavus* and *A. parasiticus*. Aflatoxin contamination of peanuts in the field can be reduced by 77–98% with biological control through the application of nontoxigenic strains of these species, which competitively exclude native aflatoxin-producing strains from developing peanuts. In this study, viable peanut seeds were artificially wounded and inoculated with field soil containing natural fungal populations that were supplemented with conidia of nontoxigenic *A. flavus* NRRL 21882 (*niaD* nitrate-nonutilizing mutant) and *A. parasiticus* NRRL 21369 (conidial color mutant). Increasing soil densities of applied nontoxigenic strains generally resulted in an increase in the incidence of seed colonization by applied nontoxigenic strains, a decrease in seed colonization by native *A. flavus* and *A. parasiticus*, and a decrease in aflatoxin concentration in seeds. Reduction of aflatoxins in peanut seeds depended on both the density and the aflatoxin-producing potential of native populations and on the fungal strain used for biological control. Wild-type strain *A. flavus* NRRL 21882 and its *niaD* mutant were equally effective in reducing aflatoxins in peanuts, indicating that nitrate-nonutilizing mutants, which are easily monitored in the field, can be used for evaluating the efficacy of biocontrol strains.

Keywords: aflatoxin; *Arachis hypogaea*; *Aspergillus flavus*; *Aspergillus parasiticus*; biological control; groundnut; peanut

Introduction

The carcinogenic aflatoxins are produced by *Aspergillus flavus* Link and *A. parasiticus* Speare, fungi belonging to *Aspergillus* section *Flavi* that commonly invade peanuts, maize, cottonseed, and tree nuts before harvest and during storage (Payne 1998). The cost of regulating aflatoxins in commodities for domestic use and foreign export in the United States is substantial (Robens and Cardwell 2005); losses to the peanut industry alone amount to approximately \$25 million annually (Lamb and Sternitske 2001). In regions of the world where aflatoxins are not highly regulated, consumption of aflatoxin-contaminated food is associated with acute liver damage, liver cancer, immunosuppression, and nutritional interference (Williams et al. 2004). Of the four

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types of aflatoxins (B_1 , B_2 , G_1 , G_2), aflatoxin B_1 is the most toxic to animals (Cullen and Newberne 1994).

Aspergillus flavus, the dominant aflatoxigenic species in crops, has been divided into two morphotypes: the L strain, which produces large sclerotia $>400\text{ }\mu\text{m}$ in diameter, and the S strain, also described as *A. parvisclerotigenus* (Saito & Tsuruta) Frisvad & Samson (Frisvad, Skouboe, and Samson 2005), which produces numerous small sclerotia $<400\text{ }\mu\text{m}$ (Cotty 1989). The L strain produces aflatoxins B_1 and B_2 and is extremely variable in aflatoxin production, with isolates ranging from nonaflatoxigenic to highly aflatoxigenic (Horn and Dorner 1999). The S strain of *A. flavus* in the United States produces only B aflatoxins (Horn and Dorner 1999), but some isolates in South America, Africa, Australia, and Southeast Asia produce both B and G aflatoxins (Saito and Tsuruta 1993; Cotty and Cardwell 1999; Geiser, Dorner, Horn, and Taylor 2000; Barros, Torres, and Chulze 2005). The S strain generally produces higher levels of aflatoxins than the L strain (Cotty 1989; Horn and Dorner 1999). *Aspergillus parasiticus* is found more frequently in peanuts than in aerial crops, although the frequency of occurrence in peanuts is still much less than that of *A. flavus*. The species produces aflatoxins G_1 and G_2 in addition to the B aflatoxins and at generally high total concentrations (Horn et al. 1996). Other species from section *Flavi* that occur less frequently in crops include *A. caelatus* B.W. Horn, *A. tamarii* Kita, and *A. alliaceus* Thom & Church; none of these species produces aflatoxins.

Soil is a reservoir for aflatoxigenic fungi and serves as a source of primary inoculum for infection of crops. Because peanuts fruit underground, developing pods are in direct contact with soil populations. Peanuts are most susceptible to invasion by *A. flavus* and *A. parasiticus* under conditions of drought stress and elevated soil temperatures (Sanders, Hill, Cole, and Blankenship 1981; Blankenship, Cole, Sanders, and Hill 1984). These conditions also favor pod and seed damage by arthropods, notably the lesser cornstalk borer, *Elasmopalpus lignosellus* (Zeller) (Lepidoptera: Pyralidae), in the United States (Lynch 1984) and beetle larvae, termites, and millipedes in tropical regions (Lynch and Mack 1995). Damaged peanut seeds contain the highest concentrations of aflatoxins (Sanders, Cole, Blankenship, and Hill 1985; Dowell, Dorner, Cole, and Davidson 1990).

Biological control using nontoxigenic strains of *A. flavus* and *A. parasiticus* effectively reduces aflatoxin contamination of peanuts by 77–98% at harvest during drought years (Dorner 2005). Conidia of nontoxigenic strains typically are coated onto a substrate such as rice or barley, which then is applied to the soil surface after development of the peanut canopy (Cole and Dorner 2001). In the humid conditions beneath the canopy, nontoxigenic strains rapidly colonize the grain and sporulate profusely on its surface. Conidia are subsequently washed by rainfall into the soil, where they remain within the pod zone of peanuts. Applied nontoxigenic strains when present at overwhelmingly high soil densities are assumed to competitively exclude native toxigenic strains from invasion sites on peanut seeds (Dorner, Cole, and Blankenship 1992), though the mechanism responsible for aflatoxin reduction is poorly understood.

Biocontrol studies often require the monitoring of applied nontoxigenic strains in soil and crops. Identification of applied wild-type strains by mycotoxin analysis (Dorner and Cole 2002) or vegetative compatibility analysis (Cotty 1994) is labor intensive. Nitrate-nonutilizing (*nit*) mutants readily invade crops where nitrogen sources are complex (Cotty and Bayman 1993) and have the advantage of ease in

identification. However, a direct comparison of the effectiveness of wild-type strains and their respective *nit* mutants in inhibiting aflatoxin formation has not been examined.

Past biological control studies have relied on bulk analyses of peanuts to measure seed colonization and aflatoxin content (Dorner et al. 1992; Dorner, Cole, and Blankenship 1998; Dorner and Cole 2002; Dorner and Horn 2007) and as a consequence, information was lost concerning the variability associated with competition within individual seeds. Horn (2005) developed a laboratory procedure in which viable peanut seeds are individually wounded and the wounds are inoculated with soil from the field. Inoculation of peanut seeds with natural soil populations of aflatoxigenic fungi has provided detailed information on the effects of temperature, seed water activity, and soil population density on seed colonization (Horn 2005, 2006). For the present research, artificially wounded seeds were individually inoculated with field soil containing natural fungal populations to examine (1) the effects of soil density of applied nontoxigenic *A. flavus* and *A. parasiticus* on aflatoxin reduction and (2) the relative effectiveness of an *A. flavus* wild-type strain and its *nit* mutant as biocontrol agents.

Materials and methods

Fungal strains

Mutants of nontoxigenic *A. flavus* and *A. parasiticus* were used to distinguish them from native wild-type strains in the experiments. *Aspergillus flavus* NRRL 21882, the active component of the biocontrol formulation afla-guard[®], was originally isolated in 1991 from a peanut seed in Dawson, Georgia, USA (Dorner 2005). A nitrogen nonutilizing (*nit*) mutant, *niaD*, was created for the purposes of the present study as described by Horn and Greene (1995). *Aspergillus parasiticus* NRRL 21369 was ultimately derived from NRRL 5862 (=SU-1), which was UV mutated to produce the orange-brown conidial color mutant NRRL 6111 with reduced aflatoxin production (Bennett and Goldbatt 1973). NRRL 6111 was then further UV mutated to create the nontoxigenic NRRL 21369 (Dorner et al. 1998). Both *A. flavus* NRRL 21882 (wild type) and *A. parasiticus* NRRL 21369 have been effectively used in biological control for the reduction of aflatoxins in peanuts (Dorner et al. 1998; Dorner and Cole 2002; Dorner and Horn 2007).

Experiments on aflatoxin reduction by applied nontoxigenic strains at different soil population densities

Collection and preparation of field soil

Soil was collected from the same approximate location in a peanut field near Graves, Terrell Co., Georgia, on 14 May 2003 (NRRL 21369, experiment 1), 10 Oct 2003 (NRRL 21882 *niaD*, experiment 1), 28 Oct 2005 (NRRL 21369, experiment 2), and 31 Mar 2006 (NRRL 21882 *niaD*, experiment 2). Five soil samples (approximately 1 kg each) were collected from the top 5 cm, air dried for 7–10 days at room temperature, sieved through no. 12 and 20 standard sieves, then pooled and thoroughly mixed. Sieved soil (203.3 g) was added to sterile glass jars and stored at 5°C. Soil samples were used within 20 days for enumeration of populations and

inoculation of peanut seeds. Experiments using *A. flavus* NRRL 21882 *niaD* and *A. parasiticus* NRRL 21369 were each performed twice.

Native Aspergillus populations in soil

Soil population densities of native fungi were determined by removing 3.3 g soil from each jar and vortexing in 10 mL of 0.2% water agar. Section *Flavi* species (*A. flavus* L strain, *A. flavus* S strain, *A. parasiticus*, *A. tamarii*, *A. caelatus*, and *A. alliaceus*) and section *Nigri* (species not separated) were enumerated by diluting 2 mL of soil suspension with 8 mL water agar, plating on a modified dichloran-rose bengal medium (mDRB) (0.2 mL per plate; 10 plates per jar), and incubating for 3–4 days at 37°C (Horn and Dorner 1998). Total populations of filamentous fungi were enumerated by diluting 0.2 mL of soil suspension with 100 mL water agar, plating on an unmodified dichloran-rose bengal medium (DRB) (0.2 mL per plate; 10 plates per jar), and incubating for 5 days at 25°C (Horn, Dorner, Greene, Blankenship, and Cole 1994). All fungal densities were adjusted for the added water in the preparation of the soil paste used to inoculate peanut seeds (see below).

To measure the aflatoxigenicity of section *Flavi* populations in soil, 100 colonies of *A. flavus* L strain, 50 colonies of *A. flavus* S strain, and 50 colonies of *A. parasiticus* (when available) were randomly chosen from the mDRB soil dilution plates in each experiment. Isolates were single spored by dilution plating onto DRB plates, and germlings (20 h at 30°C) were transferred to slants containing Czapek agar (Cz) (Raper and Fennell 1965).

Introduced nontoxigenic mutants in soil

Conidia were obtained from slant cultures grown 14 days at 30°C on either ammonium agar (Horn and Greene 1995) with 400 g sucrose added to each liter (*A. flavus* NRRL 21882 *niaD*) or on Cz with 400 g added sucrose (*A. parasiticus* NRRL 21369). Glass beads were coated with conidia for effective mixing of conidia in soil. For each fungal strain, 2.5 g of sterile glass beads (100–170 U.S. sieve; 90–150 µm diameter) was added to each of four slants and shaken, then combined in a sterile glass vial before drying over desiccant in a desiccator jar. Glass beads were added to glass jars containing sieved soil and thoroughly mixed; preliminary subsampling from individual jars (ten 3.3-g subsamples per jar; plated as described above) showed a homogenous distribution of conidia. Amounts of beads (mg) added to jars were: 0 (control), 0.6–1.7, 1.4–7.2, 5.2–50.9, 11.9–70.0, 54.5–180.6, 170.7–370.6 and 264.1–573.3 for experiments 1 and 2. Weights of glass beads were adjusted to produce similar soil densities of applied nontoxigenic strains between experiments based on preliminary soil dilutions.

Nontoxigenic strains were enumerated by suspending 33 g soil from each jar in 100 mL water agar and dilution plating as described for native soil populations. *Aspergillus flavus* NRRL 21882 *niaD* was identified by randomly transferring 50 colonies distributed among the mDRB plates from each jar to slants of Cz with a sole nitrogen source of NO₃, which resulted in thin growth and sparse sporulation by the *niaD* mutant. *Aspergillus parasiticus* NRRL 21369 was identified directly from plates by its orange–brown conidia.

Peanut seed inoculation

Viable peanut seeds were prepared and inoculated with soil according to the procedure of Horn (2005). Briefly, undamaged peanut pods (cultivar Georgia Green) were surface sterilized for 2 min with 2.0% sodium hypochlorite and the seeds were aseptically removed. Seeds were placed in four-sectioned plates (four seeds per plate) and incubated for 7 days at 37°C in covered desiccator jars over salt solution ($A_w = 0.93$), resulting in seeds equilibrating to a water activity of 0.92. Seeds without visible fungal growth following incubation were aseptically wounded on the cotyledon with a cork borer and dissecting needle, then the wounds were inoculated with 6.8 ± 1.55 mg (\pm SD, $n=60$) of a near-saturated soil paste (33 g soil plus 8.0 mL distilled water). Forty wounded and inoculated seeds per jar of soil were incubated an additional 14 days under the same conditions. In addition, 20 seeds were wounded but not inoculated for each experiment. Of the 140 total uninoculated seeds, none showed fungal contamination at the end of the experiments.

Fungi colonizing peanut seeds

Colonized peanut seeds were examined with the stereomicroscope at 4, 7 and 14 days following soil inoculation (Horn 2006). With the exception of *A. alliaceus*, wild-type section *Flavi* species could not be distinguished on peanut seeds. Conidia were therefore transferred from one to 10 regions of the seed (depending upon the extent of colonization) to Cz slants for identification. As with the soil dilution plates, *A. flavus* NRRL 21882 *niaD* was recognized by its growth pattern following transfer to Cz slants and the orange–brown conidial mutant *A. parasiticus* NRRL 21369 was identified directly from the peanut seed. One wild-type isolate each of *A. flavus* L strain, *A. flavus* S strain and *A. parasiticus* from each seed (when present) were single spored as described above.

Experiments comparing aflatoxin reduction by a nontoxigenic wild-type strain and its *niaD* mutant

Experiments of wild-type *A. flavus* NRRL 21882 verses its *niaD* mutant were performed twice. Soil was collected from the peanut field near Graves, Terrell Co., Georgia, on 10 Oct 2003 (experiment 1) and 4 June 2004 (experiment 2). Jars of soil were separately inoculated with wild-type *A. flavus* NRRL 21882 and its *niaD* mutant as previously described, with bead amounts of each strain added to soil as follow: 0 (control), 9.4–24.0, 75.8–330.0 and 410.4–1781.7 mg. Soil densities of *A. flavus* NRRL 21882 wild type and *niaD* were estimated by the increase in colony numbers over the initial native population density of the species. Peanut seeds were prepared and inoculated with soil as previously described.

Aflatoxin analyses

Aspergillus flavus and *A. parasiticus* isolates from soil and peanut seeds were each grown in two vials containing yeast-extract sucrose broth, cultures were extracted with chloroform, and aflatoxins were analyzed by liquid chromatography as

described in Horn and Dorner (1999). Mean aflatoxin values of replicate vial cultures were used in all calculations.

Aflatoxins in seeds were quantified by weighing individual seeds to the nearest 0.01 g and adding each seed to a test tube containing 5 mL of methanol. The tube was vortexed for 30 s and left in the dark for 24 h. The test tube was then vortexed for another 30 s, and the contents were gravity filtered through Whatman #4 filter paper. A 0.5-mL aliquot of filtrate was mixed with 0.5 mL of acetonitrile, applied to an aluminum oxide cleanup cartridge, and analyzed by liquid chromatography (Sobolev and Dorner 2002). Individual aflatoxins were quantified by the external standard method on a ng/g basis as if each seed weighed 1 g. Amounts were corrected by dividing the result by the pre-extraction weight of the seed.

Statistics

Linear regressions were used to examine the effects of soil density of applied nontoxigenic *A. flavus* NRRL 21882 *niaD* and *A. parasiticus* NRRL 21369 [$\log(\text{CFU/g} + 1)$] on percent seed infection by nontoxigenic strains, percent seed infection by native aflatoxigenic isolates, and aflatoxin B₁ (ppm) in peanut seeds. Pearson product-moment correlations were performed between aflatoxin B₁ produced in vial cultures by wild-type isolates from peanut seeds and aflatoxin B₁ in the same peanut seeds. In instances where more than one aflatoxigenic species occurred on a peanut seed, mean aflatoxin B₁ values among species were used. All regressions and correlations met the assumptions of a normal distribution and constant variance. Data on aflatoxin B₁ concentrations in different seed categories were not normally distributed, even after various transformations; therefore, Kruskal–Wallis ANOVA on ranks was used. Regression, correlation, and nonparametric ANOVA analyses were performed using SigmaStat 3.5 (Jandel Scientific, San Rafael, CA).

Wild-type *A. flavus* NRRL 21882 and its *niaD* mutant were compared according to the effect of soil density [$\log(\text{CFU/g} + 1)$] on peanut seed concentrations of aflatoxin B₁ and G₁ (ppm). Analyses of covariance (ANCOVA) with soil density as the covariant were performed using SAS statistical package version 8 (SAS Institute, Cary, NC).

Results

Native Aspergillus populations in soil

Aspergillus flavus L strain consistently showed the highest population density of the aflatoxigenic species within section *Flavi* in soil samples collected for inoculating wounded peanut seeds in experiments (Table 1). The S strain of *A. flavus* and *A. parasiticus* were less abundant. Nonaflatoxigenic *A. caelatus*, *A. tamaraii*, and *A. alliaceus* from section *Flavi* also were present, and combined species from section *Nigri* were often abundant. There were considerable differences in population densities among soil samples from the same general location in the peanut field due to the effects of sampling error and possible changes in populations over time.

Populations of *A. flavus* L strain in soil showed high diversity in the amount of aflatoxin B₁ produced by isolates (Figure 1). The majority of isolates for all soil collection dates produced aflatoxins. Soil collected 28 Oct 2005 for experiment 2

Table 1. Population densities (CFU/g) of native *Aspergillus* species in soil paste used for inoculating peanut seeds.^a

Treatment ^c	<i>Aspergillus</i> species ^b							Total filamentous fungi ($\times 10^3$)
	F-L	F-S	P	C	T	A	N	
NRRL 21882 <i>niaD</i> exp. 1	1043 \pm 201	33 \pm 24	82 \pm 25	63 \pm 22	12 \pm 9	435 \pm 77	1264 \pm 56	110 \pm 13
NRRL 21882 <i>niaD</i> exp. 2	4704 \pm 536	14 \pm 26	1054 \pm 413	835 \pm 238	120 \pm 64	1181 \pm 435	1132 \pm 276	244 \pm 40
NRRL 21369 exp. 1	111 \pm 30	17 \pm 8	87 \pm 24	59 \pm 22	8 \pm 8	73 \pm 13	904 \pm 135	58 \pm 14
NRRL 21369 exp. 2	1217 \pm 309	14 \pm 15	322 \pm 117	173 \pm 72	3 \pm 10	1330 \pm 295	5097 \pm 543	214 \pm 3

^aMean \pm SD ($n = 8$).^bAbbreviations: F-L, *A. flavus* L strain; F-S, *A. flavus* S strain; P, *A. parasiticus*; C, *A. caelatus*; T, *A. tamarii*; A, *A. alliaceus*; N, section *Nigri* (combined species).^cSoil samples were collected on the following dates: NRRL 21882 *niaD* experiment 1 (10 Oct 2003), NRRL 21882 *niaD* experiment 2 (31 Mar 2006), NRRL 21369 experiment 1 (14 May 2003), and NRRL 21369 experiment 2 (28 Oct 2005).

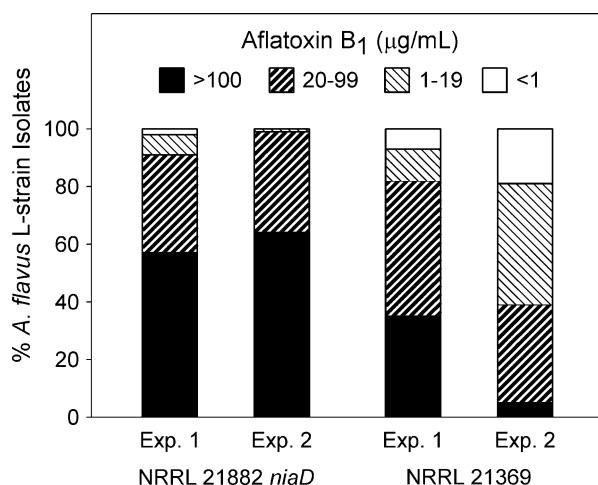


Figure 1. Production of aflatoxin B₁ by *A. flavus* L-strain isolates obtained from soil samples that were used to inoculate wounded peanut seeds. Soil samples were collected on the following dates: NRRL 21882 *niaD* experiment 1 (10 Oct 2003), NRRL 21882 *niaD* experiment 2 (31 Mar 2006), NRRL 21369 experiment 1 (14 May 2003), and NRRL 21369 experiment 2 (28 Oct 2005).

using NRRL 21369 contained the least aflatoxigenic population, with 19% of isolates producing <1 µg/mL aflatoxin B₁ and only 5% producing >100 µg/mL. *Aspergillus flavus* S strain and *A. parasiticus* showed less variation in aflatoxin production than *A. flavus* L strain. In isolates from all experiments combined, *A. flavus* S strain produced either very low (0.6 ± 1.1 µg/mL; \pm SD, $n = 31$) or very high (241 ± 103.3 µg/mL; $n = 31$) concentrations of aflatoxin B₁. The G aflatoxins were not detected in any of the S-strain isolates. In contrast, *A. parasiticus* consistently produced high concentrations of total aflatoxins, with the exception of 10 isolates that accumulated *O*-methylsterigmatocystin, the immediate precursor to aflatoxin B₁, instead of aflatoxins (Horn et al. 1996). Aflatoxigenic isolates of *A. parasiticus* from all experiments ($n = 190$) produced 96 ± 63.6 µg/mL aflatoxin B₁ and 101 ± 68.1 µg/mL aflatoxin G₁.

Experiments on aflatoxin reduction by applied nontoxigenic strains at different soil population densities

A *nit* mutant of *A. flavus* NRRL 21882 and a conidial color mutant of *A. parasiticus* NRRL 21369 were used so that applied nontoxigenic strains could be identified in soil and peanut seeds. Figures 2 and 3 show the effects of increasing concentrations of NRRL 21882 *niaD* and NRRL 21369 on the (1) incidence of seed colonization by applied nontoxigenic strains; (2) incidence of seed colonization by native aflatoxin-producing species (*A. flavus* and/or *A. parasiticus*); and (3) aflatoxin B₁ concentration in seeds. Increasing soil densities of applied nontoxigenic strains resulted in increasing incidences of seed colonization ($n = 8$) by those strains in NRRL 21882 *niaD* in experiment 1 ($R^2 = 0.85$; $P = 0.001$) and experiment 2 ($R^2 = 0.83$; $P < 0.01$) and in NRRL 21369 experiment 1 ($R^2 = 0.80$; $P < 0.01$) and experiment 2 ($R^2 = 0.80$;

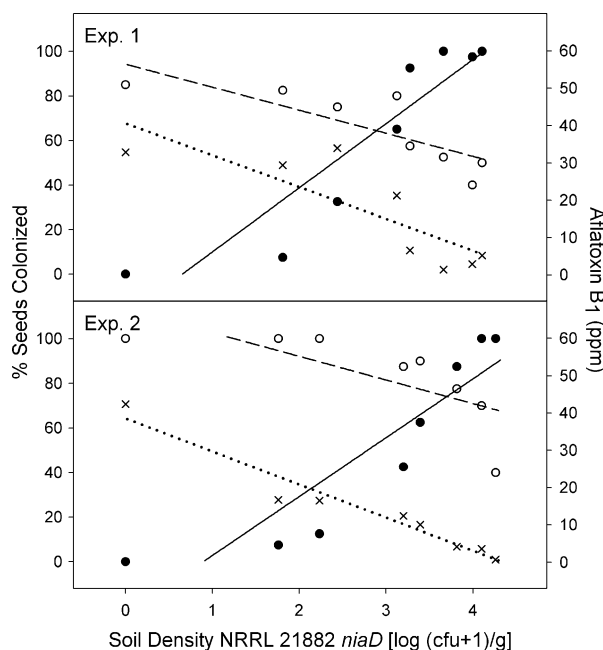


Figure 2. Linear regressions showing the effects of soil densities of applied nontoxigenic *A. flavus* NRRL 21882 *niaD* on the incidence of peanut seed colonization by applied nontoxigenic strains (—●—), the incidence of peanut seed colonization by native aflatoxigenic species (*A. flavus* and *A. parasiticus*) (---○---), and the concentration of aflatoxin B₁ in peanut seeds (···×···). Data from two experiments are shown; all datum points represent means of 40 seeds. All regressions are significant ($R^2=0.55\text{--}0.94$; $P<0.05$).

$P<0.01$). The incidence of native aflatoxigenic species colonizing seeds ($n=8$) concomitantly decreased in NRRL 21882 *niaD* experiment 1 ($R^2=0.67$; $P=0.01$) and experiment 2 ($R^2=0.55$; $P<0.05$) and in NRRL 21369 experiment 1 ($R^2=0.91$; $P<0.001$). No significant decrease in native aflatoxigenic species was observed in NRRL 21369 experiment 2 ($R^2=0.36$; $P>0.05$). Increasing soil densities of applied nontoxigenic strains also resulted in decreases in seed aflatoxin B₁ ($n=8$) in NRRL 21882 *niaD* experiment 1 ($R^2=0.68$; $P=0.01$) and experiment 2 ($R^2=0.94$; $P<0.0001$) and in NRRL 21369 experiment 1 ($R^2=0.80$; $P<0.01$). No significant decrease in aflatoxin B₁ was observed in NRRL 21369 experiment 2 ($R^2=0.22$; $P>0.05$).

Aflatoxin B₁ concentrations were also examined in peanut seeds that were categorized according to the fungal colonists present (Figure 4). Aflatoxin B₁ concentrations were significantly different as follow: seeds with native aflatoxigenic species only > seeds with a mixture of native aflatoxigenic species and the applied nontoxigenic strain > seeds with applied nontoxigenic strain only. The only exception was *A. parasiticus* NRRL 21369 experiment 2 in which aflatoxin B₁ in seeds with native aflatoxigenic species was not significantly different from aflatoxin B₁ in seeds with a mixture of native aflatoxigenic species and NRRL 21369.

The aflatoxin-producing potential of native *A. flavus* and *A. parasiticus* isolates from seeds, as measured in liquid culture, greatly influenced the aflatoxin B₁

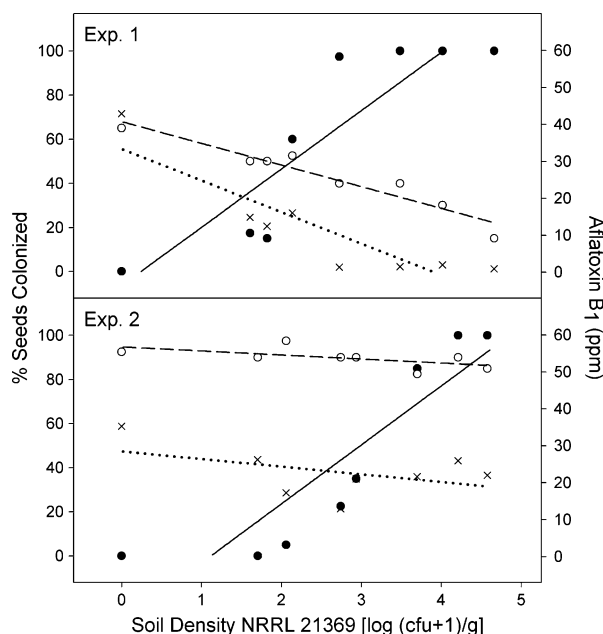


Figure 3. Linear regressions showing the effects of soil densities of applied nontoxicogenic *A. parasiticus* NRRL 21369 on the incidence of peanut seed colonization by applied nontoxicogenic strains (—●—), the incidence of peanut seed colonization by native aflatoxigenic species (*A. flavus* and *A. parasiticus*) (—○—), and the concentration of aflatoxin B₁ in peanut seeds (···×···). Data from two experiments are shown; all datum points represent means of 40 seeds. All regressions are significant ($R^2=0.80-0.91$; $P<0.01$), except for regressions in experiment 2 showing peanut seed colonization by native aflatoxigenic species ($R^2=0.36$; $P>0.05$) and aflatoxin B₁ concentration in peanut seeds ($R^2=0.22$; $P>0.05$).

concentration in peanut seeds. There was a statistically significant correlation ($n=320$; $P<0.0001$) between aflatoxigenicity of isolates and aflatoxin B₁ in seeds in NRRL 21882 *niaD* experiment 1 ($r=0.60$) and experiment 2 ($r=0.33$) and in *A. parasiticus* NRRL 21369 experiment 1 ($r=0.60$) and experiment 2 ($r=0.42$).

Experiments comparing aflatoxin reduction by a nontoxicogenic wild-type strain and its *niaD* mutant

Native populations in soil samples consisted of 1043 and 481 CFU/g of *A. flavus* L strain and 82 and 49 CFU/g *A. parasiticus* for experiments 1 and 2, respectively. No significant differences (ANCOVA, $P>0.05$) in aflatoxin reduction in peanut seeds were found between wild type and the *niaD* of nontoxicogenic *A. flavus* NRRL 21882 in experiment 1 ($F_{(1,5)}=0.47$ for B₁; $F_{(1,5)}=0.78$ for G₁) and experiment 2 ($F_{(1,5)}=0.41$ for B₁; $F_{(1,5)}=0.43$ for G₁).

Discussion

The present research using individual peanut seeds inoculated with soil containing native populations of aflatoxigenic fungi affirms the principle of competitive

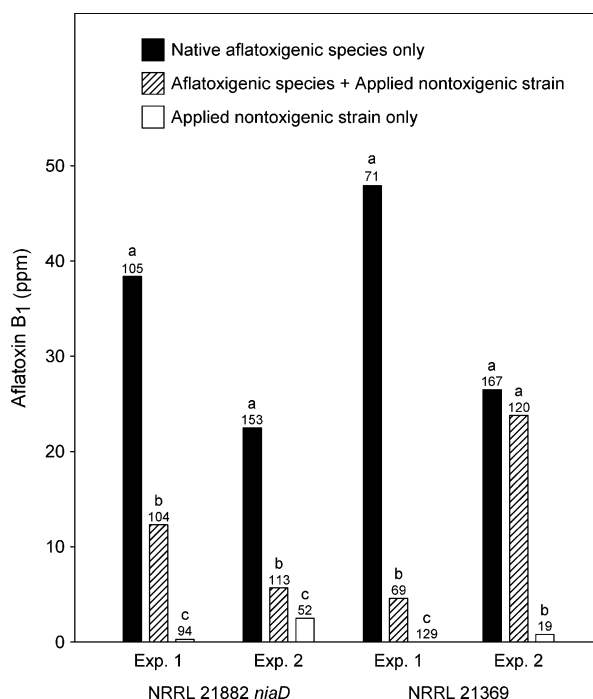


Figure 4. Aflatoxin B₁ concentrations in peanut seeds colonized by native aflatoxigenic species only (*A. flavus* and *A. parasiticus*), a mixture of native aflatoxigenic species and the applied nontoxigenic strain, and the applied nontoxigenic strain only. Mean values are shown, but statistics (Kruskal–Wallis ANOVA on ranks) are based on median values. Number of seeds is indicated at the top of each bar. Bars with different letters within each experiment are significantly different ($P < 0.05$).

exclusion by applied biocontrol strains to reduce aflatoxin contamination. In experiments 1 and 2 using *A. flavus* NRRL 21882 *niaD*, increasing soil densities of the nontoxigenic strain resulted in an increase in percent seed colonization by NRRL 21882 *niaD*, a decrease in seed colonization by native *A. flavus* and *A. parasiticus*, and a concomitant decrease in seed aflatoxin B₁ (Figure 2). A similar decrease in colonization by native strains as well as seed aflatoxin B₁ was observed with *A. parasiticus* NRRL 21369 in experiment 1 (Figure 3). These results differ from those of Dorner et al. (1998), who found no significant relationships between soil inoculum rate of applied nontoxigenic *A. flavus*/*A. parasiticus* and colonization of peanut seeds by native strains, despite significant decreases in aflatoxins. Subsequent studies of biocontrol in peanuts using *A. flavus* NRRL 21882 showed a decrease in seed colonization by native *A. flavus* and *A. parasiticus* at one soil treatment density (Dorner and Horn 2007; Dorner 2008).

Soil application with *A. parasiticus* NRRL 21369 in experiment 2 (Figure 3) proved to be the exception, with increasing soil densities of the nontoxigenic strain having no significant effect on the incidence of native aflatoxigenic fungi and seed aflatoxin B₁ despite an increase in the seed incidence of NRRL 21369. In addition, aflatoxin B₁ concentrations in seeds colonized solely by native *A. flavus* and/or *A. parasiticus* were not significantly different from concentrations in seeds colonized by

a mixture of native strains and NRRL 21369. This lack of efficacy was coupled with the observation that soil populations of *A. flavus* L strain in experiment 2 were less aflatoxigenic than those used for experiment 1. However, the soil density of native *A. flavus* L strain in experiment 2 (1217 CFU/g) was much higher than in experiment 1 (111 CFU/g). If the soil densities are adjusted for isolates that are moderately to highly aflatoxigenic (>20 ppm in liquid culture), then experiment 2 with 986 CFU/g had a much higher aflatoxin-producing potential than experiment 1 (103 CFU/g). The present research showed that aflatoxin contamination of individual peanut seeds was positively correlated with the capacity of fungal isolates from seeds to produce aflatoxins in liquid culture.

Several field studies have demonstrated that *A. parasiticus* NRRL 21369 is less effective in reducing aflatoxins than *A. flavus* NRRL 21882 (Dorner, Cole, and Wicklow 1999; Dorner and Horn 2007). Horn et al. (1994) also showed that *A. parasiticus* NRRL 6111, the progenitor strain of NRRL 21369, is less invasive of peanuts than wild-type *A. flavus* even under conditions of high soil population densities of NRRL 6111. Therefore, the low competitive ability of *A. parasiticus* NRRL 21369 coupled with a relatively high aflatoxin-producing potential of the native soil population in experiment 2 resulted in no significant reduction of aflatoxins. The more aggressive *A. flavus* NRRL 21882, in contrast, was effective in reducing aflatoxins resulting from soil populations with both high densities and aflatoxigenicities.

Aspergillus flavus NRRL 21882 and its *niaD* mutant were equally effective in reducing aflatoxins in peanuts. Therefore, the use of the mutant for identification purposes in these experiments was an accurate assessment of the wild-type strain for biological control. These data also suggest that in field trials, *nit* mutants would be effective for the monitoring of applied nontoxigenic strains in soil and crops.

This study shows the necessity of evaluating nontoxigenic strain performance before implementing biocontrol, as well as the importance of the aflatoxin-producing potential of the targeted native population on the rate of biocontrol application for effective control of aflatoxins. Variability in aflatoxin contamination of individual viable peanut seeds inoculated with soil containing natural fungal populations in the study was high overall, a characteristic of aflatoxin research at all levels of sampling and analysis (Whitaker, Dowell, Hagler, Giesbrecht, and Wu 1994). Nevertheless, the peanut seed assay has the advantage of greatly increasing sample numbers as well as utilizing the unit of study (seed) most relevant to fungal infection. This assay previously has demonstrated that the optimum temperature for seed colonization is 30–37°C for *A. flavus* and 22°C for *A. parasiticus* and that the optimum seed water activity for both species is 0.92–0.96 (Horn 2005). In addition, the effect of soil population density and fungal competition on the colonization of peanut seeds by aflatoxigenic fungi has been quantified (Horn 2006). The present study indicates that the assay is similarly useful for studying the role of fungal competition in aflatoxin reduction associated with biological control.

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References

- Barros, G., Torres, A., and Chulze, S. (2005), 'Aspergillus flavus Population Isolated from Soil of Argentina's Peanut-Growing Region. Sclerotia Production and Toxigenic Profile', *Journal of the Science of Food and Agriculture*, 85, 2349–2353.
- Bennett, J.W., and Goldblatt, L.A. (1973), 'The Isolation of Mutants of *Aspergillus flavus* and *A. parasiticus* with Altered Aflatoxin Producing Ability', *Sabouraudia*, 11, 235–241.
- Blankenship, P.D., Cole, R.J., Sanders, T.H., and Hill, R.A. (1984), 'Effect of Geocarposphere Temperature on Pre-Harvest Colonization of Drought-Stressed Peanuts by *Aspergillus flavus* and Subsequent Aflatoxin Contamination', *Mycopathologia*, 85, 69–74.
- Cole, R.J., and Dorner, J.W. (2001), 'Biological Control Formulations Containing Spores of Nontoxigenic Strains of Fungi for Toxin Control of Food Crops', U.S. Patent 6,306,386, 23 October 2001.
- Cotty, P.J. (1989), 'Virulence and Cultural Characteristics of Two *Aspergillus flavus* Strains Pathogenic on Cotton', *Phytopathology*, 79, 808–814.
- Cotty, P.J. (1994), 'Influence of Field Application of an Atoxigenic Strain of *Aspergillus flavus* on the Populations of *A. flavus* Infecting Cotton Bolls and on the Aflatoxin Content of Cottonseed', *Phytopathology*, 84, 1270–1277.
- Cotty, P.J., and Bayman, P. (1993), 'Competitive Exclusion of a Toxigenic Strain of *Aspergillus flavus* by an Atoxigenic Strain', *Phytopathology*, 83, 1283–1287.
- Cotty, P.J., and Cardwell, K.F. (1999), 'Divergence of West African and North American Communities of *Aspergillus* section *Flavi*', *Applied and Environmental Microbiology*, 65, 2264–2266.
- Cullen, J.M., and Newberne, P.M. (1994), 'Acute Hepatotoxicity of Aflatoxins', in *The Toxicology of Aflatoxins: Human Health, Veterinary, and Agricultural Significance*, eds. D.L. Eaton and J.D. Groopman, San Diego, CA: Academic Press, pp. 3–26.
- Dorner, J.W. (2005), 'Biological Control of Aflatoxin Crop Contamination', in *Aflatoxin and Food Safety*, ed. H.K. Abbas, Boca Raton, FL: CRC Press, pp. 333–352.
- Dorner, J.W. (2009), 'Development of Biocontrol Technology to Manage Aflatoxin Contamination in Peanuts', *Peanut Science*, 36, 60–67.
- Dorner, J.W., and Cole, R.J. (2002), 'Effect of Application of Nontoxigenic Strains of *Aspergillus flavus* and *A. parasiticus* on Subsequent Aflatoxin Contamination of Peanuts in Storage', *Journal of Stored Products Research*, 38, 329–339.
- Dorner, J.W., and Horn, B.W. (2007), 'Separate and Combined Applications of Nontoxigenic *Aspergillus flavus* and *A. parasiticus* for Biocontrol of Aflatoxin in Peanuts', *Mycopathologia*, 163, 215–223.
- Dorner, J.W., Cole, R.J., and Blankenship, P.D. (1992), 'Use of a Biocompetitive Agent to Control Preharvest Aflatoxin in Drought Stressed Peanuts', *Journal of Food Protection*, 55, 888–892.
- Dorner, J.W., Cole, R.J., and Blankenship, P.D. (1998), 'Effect of Inoculum Rate of Biological Control Agents on Preharvest Aflatoxin Contamination of Peanuts', *Biological Control*, 12, 171–176.
- Dorner, J.W., Cole, R.J., and Wicklow, D.T. (1999), 'Aflatoxin Reduction in Corn through Field Application of Competitive Fungi', *Journal of Food Protection*, 62, 650–656.
- Dowell, F.E., Dorner, J.W., Cole, R.J., and Davidson Jr, J.I. (1990), 'Aflatoxin Reduction by Screening Farmers Stock Peanuts', *Peanut Science*, 17, 6–8.
- Frisvad, J.C., Skouboe, P., and Samson, R.A. (2005), 'Taxonomic Comparison of Three Different Groups of Aflatoxin Producers and a New Efficient Producer of Aflatoxin B₁, Sterigmatocystin and 3-O-Methylsterigmatocystin, *Aspergillus rambellii* sp. nov.', *Systematic and Applied Microbiology*, 28, 442–453.
- Geiser, D.M., Dorner, J.W., Horn, B.W., and Taylor, J.W. (2000), 'The Phylogenetics of Mycotoxin and Sclerotium Production in *Aspergillus flavus* and *Aspergillus oryzae*', *Fungal Genetics and Biology*, 31, 169–179.
- Horn, B.W. (2005), 'Colonization of Wounded Peanut Seeds by Soil Fungi: Selectivity for Species from *Aspergillus* section *Flavi*', *Mycologia*, 97, 205–220.
- Horn, B.W. (2006), 'Relationship between Soil Densities of *Aspergillus* Species and Colonization of Wounded Peanut Seeds', *Canadian Journal of Microbiology*, 52, 951–960.

- Horn, B.W., and Dorner, J.W. (1998), 'Soil Populations of *Aspergillus* Species from section *Flavi* along a Transect through Peanut-Growing Regions of the United States', *Mycologia*, 90, 767–776.
- Horn, B.W., and Dorner, J.W. (1999), 'Regional Differences in Production of Aflatoxin B₁ and Cyclopiazonic Acid by Soil Isolates of *Aspergillus flavus* along a Transect within the United States', *Applied and Environmental Microbiology*, 65, 1444–1449.
- Horn, B.W., and Greene, R.L. (1995), 'Vegetative Compatibility within Populations of *Aspergillus flavus*, *A. parasiticus*, and *A. tamaritii* from a Peanut Field', *Mycologia*, 87, 324–332.
- Horn, B.W., Dorner, J.W., Greene, R.L., Blankenship, P.D., and Cole, R.J. (1994), 'Effect of *Aspergillus parasiticus* Soil Inoculum on Invasion of Peanut Seeds', *Mycopathologia*, 125, 179–191.
- Horn, B.W., Greene, R.L., Sobolev, V.S., Dorner, J.W., Powell, J.H., and Layton, R.C. (1996), 'Association of Morphology and Mycotoxin Production with Vegetative Compatibility Groups in *Aspergillus flavus*, *A. parasiticus*, and *A. tamaritii*', *Mycologia*, 88, 574–587.
- Lynch, R.E. (1984), 'Damage and Preference of Lesser Cornstalk Borer (Lepidoptera: Pyralidae) Larvae for Peanut Pods in Different Stages of Maturity', *Journal of Economic Entomology*, 77, 360–363.
- Lynch, R.E., and Mack, T.P. (1995), 'Biological and Biotechnical Advances for Insect Management in Peanut', in *Advances in Peanut Science*, eds. H.E. Pattee and H.T. Stalker, Stillwater, OK: American Peanut Research and Education Society, pp. 95–159.
- Lamb, M.C., and Sternitske, D.A. (2001), 'Cost of Aflatoxin to the Farmer, Buying Point, and Sheller Segments of the Southeast United States Peanut Industry', *Peanut Science*, 28, 59–63.
- Payne, G.A. (1998), 'Process of Contamination by Aflatoxin-Producing Fungi and their Impact on Crops', in *Mycotoxins in Agriculture and Food Safety*, eds. K.K. Sinha and D. Bhatnagar, New York: Marcel Dekker, pp. 279–306.
- Raper, K.B., and Fennell, D.I. (1965), *The Genus Aspergillus*, Baltimore, MD: Williams and Wilkins.
- Robens, J., and Cardwell, K.F. (2005), 'The Costs of Mycotoxin Management to the United States', in *Aflatoxin and Food Safety*, ed. H.K. Abbas, Boca Raton, FL: CRC Press, pp. 1–12.
- Saito, M., and Tsuruta, O. (1993), 'A New Variety of *Aspergillus flavus* from Tropical Soil in Thailand and its Aflatoxin Productivity', *Proceedings of the Japanese Association of Mycotoxicology*, 37, 31–36.
- Sanders, T.H., Hill, R.A., Cole, R.J., and Blankenship, P.D. (1981), 'Effect of Drought on Occurrence of *Aspergillus flavus* in Maturing Peanuts', *Journal of the American Oil Chemists' Society*, 58, 966A–970A.
- Sanders, T.H., Cole, R.J., Blankenship, P.D., and Hill, R.A. (1985), 'Relation of Environmental Stress Duration to *Aspergillus flavus* Invasion and Aflatoxin Production in Preharvest Peanuts', *Peanut Science*, 12, 90–93.
- Sobolev, V.S., and Dorner, J.W. (2002), 'Cleanup Procedure for Determination of Aflatoxins in Major Agricultural Commodities by Liquid Chromatography', *Journal of AOAC International*, 85, 642–645.
- Whitaker, T.B., Dowell, F.E., Hagler, W.M. Jr., Giesbrecht, F.G., and Wu, J. (1994), 'Variability Associated with Sampling, Sample Preparation, and Chemical Testing for Aflatoxin in Farmers' Stock Peanuts', *Journal of AOAC International*, 77, 107–116.
- Williams, J.H., Phillips, T.D., Jolly, P.E., Stiles, J.K., Jolly, C.M., and Aggarwal, D. (2004), 'Human Aflatoxicosis in Developing Countries: A Review of Toxicology, Exposure, Potential Health Consequences, and Interventions', *American Journal of Clinical Nutrition*, 80, 1106–1122.